Supplemental Material

β_2-Adrenoceptor Agonists Promote ERK1/2 Dephosphorylation in Human Airway Epithelial Cells by Canonical, cAMP-Driven Signaling Independently of β-Arrestin 2

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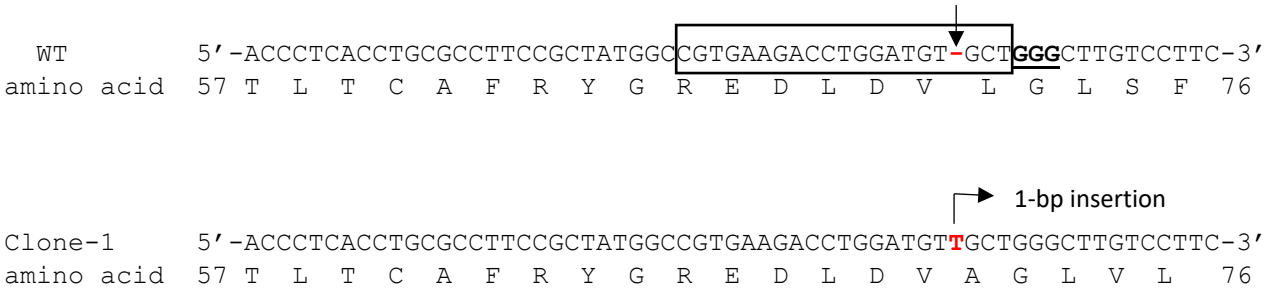
A



B

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Clone-1    1 MGEKPGTRVFKKSSPNCKLTVYLGKRDFVDHLDKVDPVDGVVLVDPDYLKDRKVFVTLTC 60

Parent    61 AFRYGREDLDVLGLSFRKDLFIATYQAFPPVPNPPRPPTRLQDRLLRKLGQHAHPFFFTV 120
Clone-1   61 AFRYGREDLDVAGLVLPQRPVHRHLPGLPPGAQPTPAPHPPAGPAAEEAGPACPPLLLHR 120

Parent   121 RMPLPSEGQGAGAGTVSGVGIPQNLPCSVTLQPGPEDTGKACGVDFEIRAFCAKSLEEKS 180
Clone-1  121 EDAPAL*---------------------------------------------------- 126

Parent   181 HKRNSVRLVIRKVQFAPEKPGPQPSAETTRHFLMSDRSLHLEASLDKELYYHGEPLNVNV 240
Clone-1  126 ------------------------------------------------------------ 126

Parent   241 HVTNNSTKTVKKIKVSVRQYADICLFSTAQYKCPVAQLEQDDQVSPSSTFCKVYTITPLL 300
Clone-1  126 ------------------------------------------------------------ 126

Parent   301 SDNREKRGLALDGKLKHEDTNLASSTIVKEGANKEVLGILVSYRVKVKLVVSRGGDVSVE 360
Clone-1  126 ------------------------------------------------------------ 126

Parent   361 LPFVLMHPKPHDHIPLPRPQSAAPETDVPVDTNLIEFDTNYATDDDIVFEDFARLRLKGM 420
Clone-1  126 ------------------------------------------------------------ 126

Parent   421 KDDDYDDQLC                                                   430
Clone-1  126 ----------                                                   126
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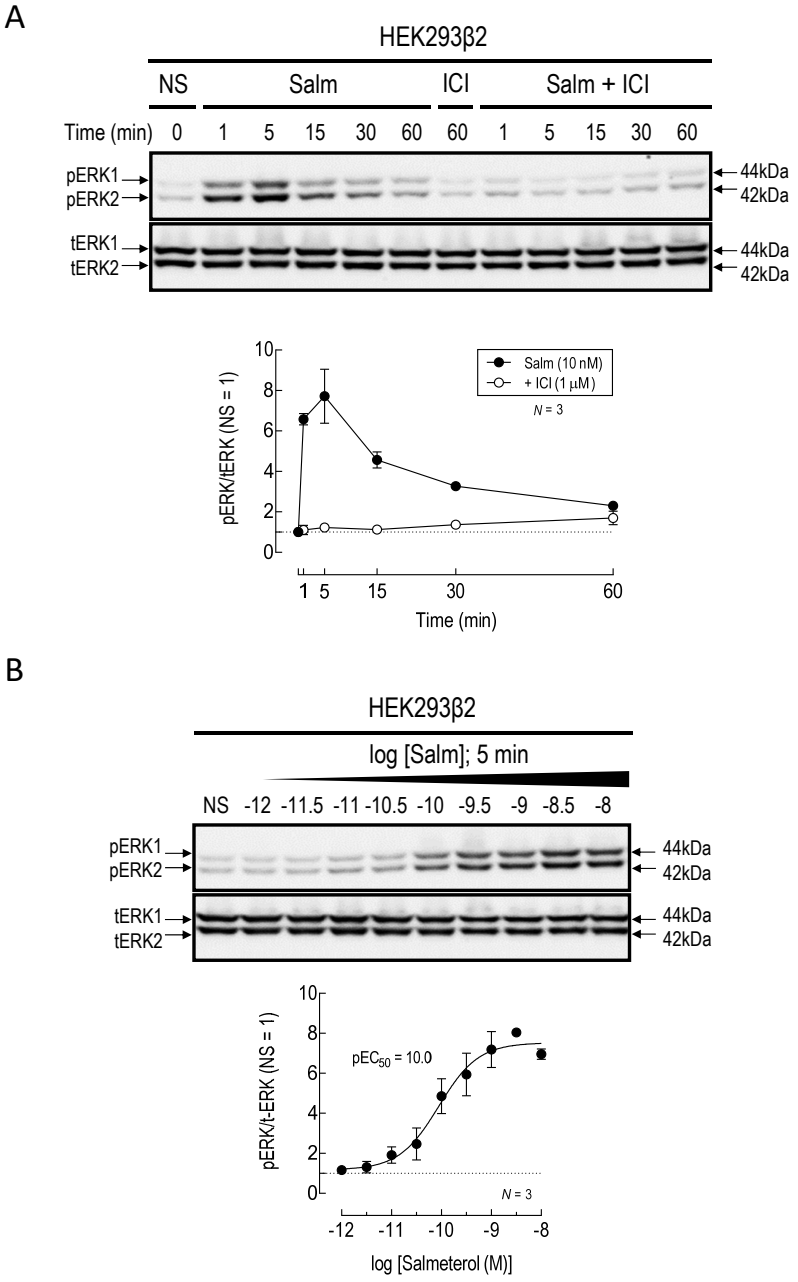
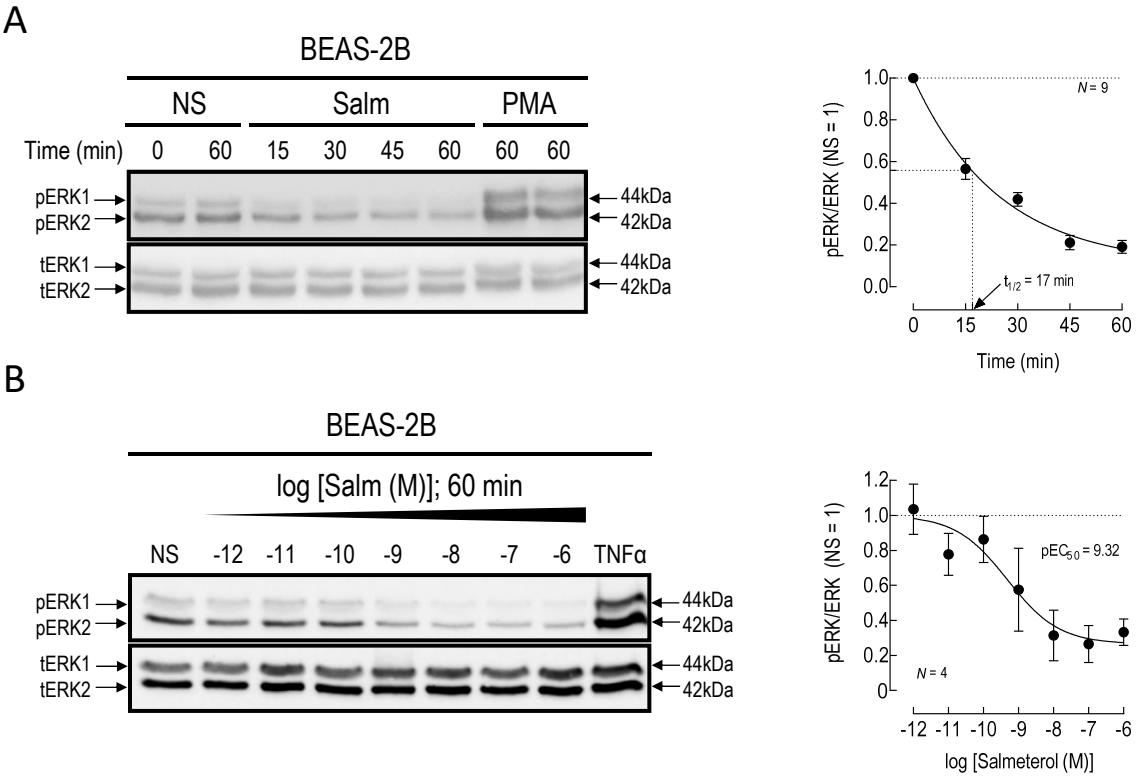
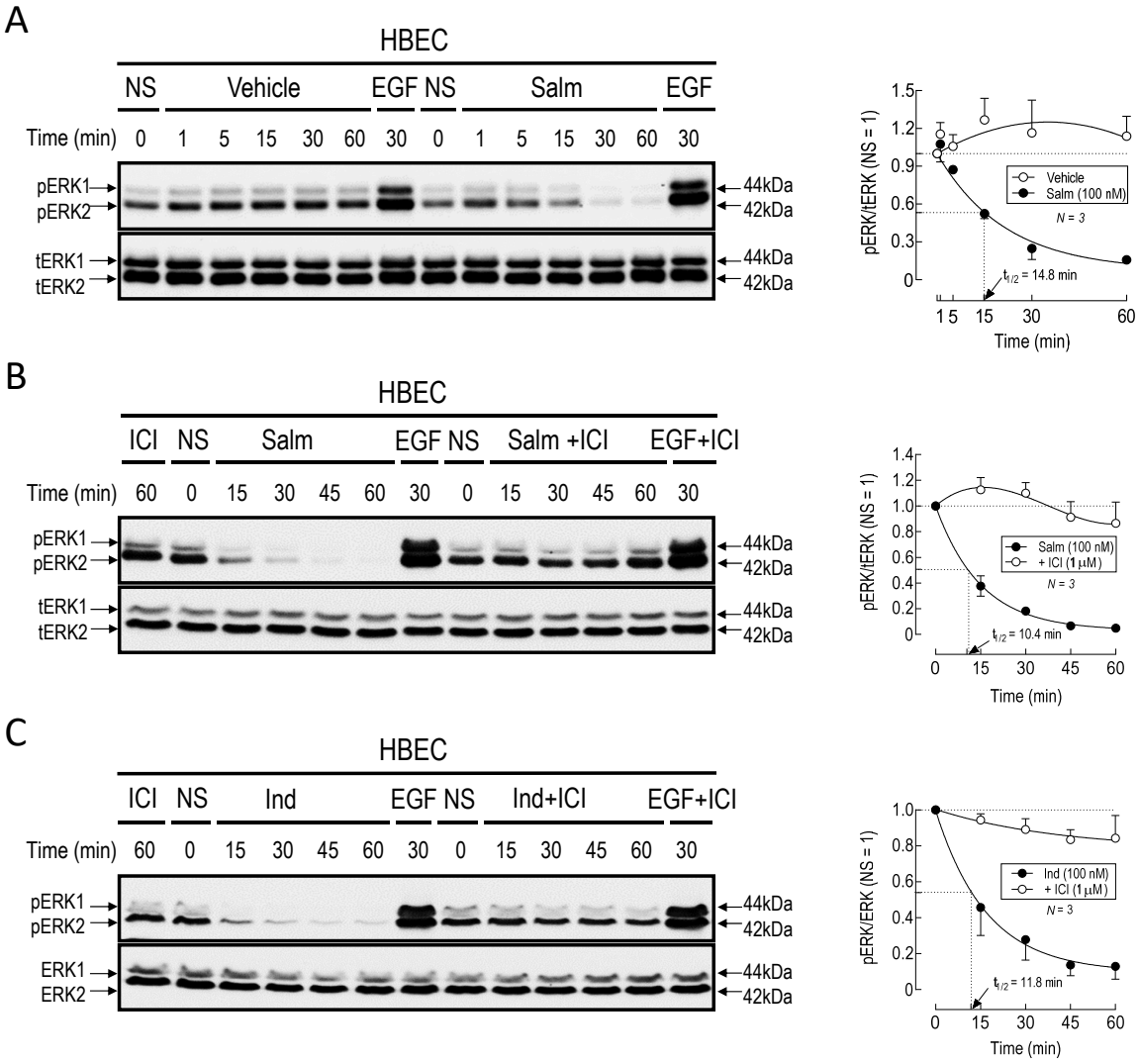
Supplemental Fig. 1. Genomic sequence of β-arrestin-2 mutant BEAS-2B cells. (**A**). The synthetic guide RNA-targeted sequence of Clone 1 is boxed and the protospacer adjacent motif sequence (GGG) is bolded and underlined. A thymine (T) insertion at the putative double strand break site is indicated by an arrow and highlighted in red. This was confirmed in all 3 alleles identified in BEAS-2B cells and resulted in a frame shift mutation and the generation of a premature stop codon (TGA). (**B**). Alignment of the deduced amino acid sequence of the β-arrestin-2 mutant. The stop codon is marked with a red asterisk. The parent β-arrestin-2 sequence refers to isoform 4.



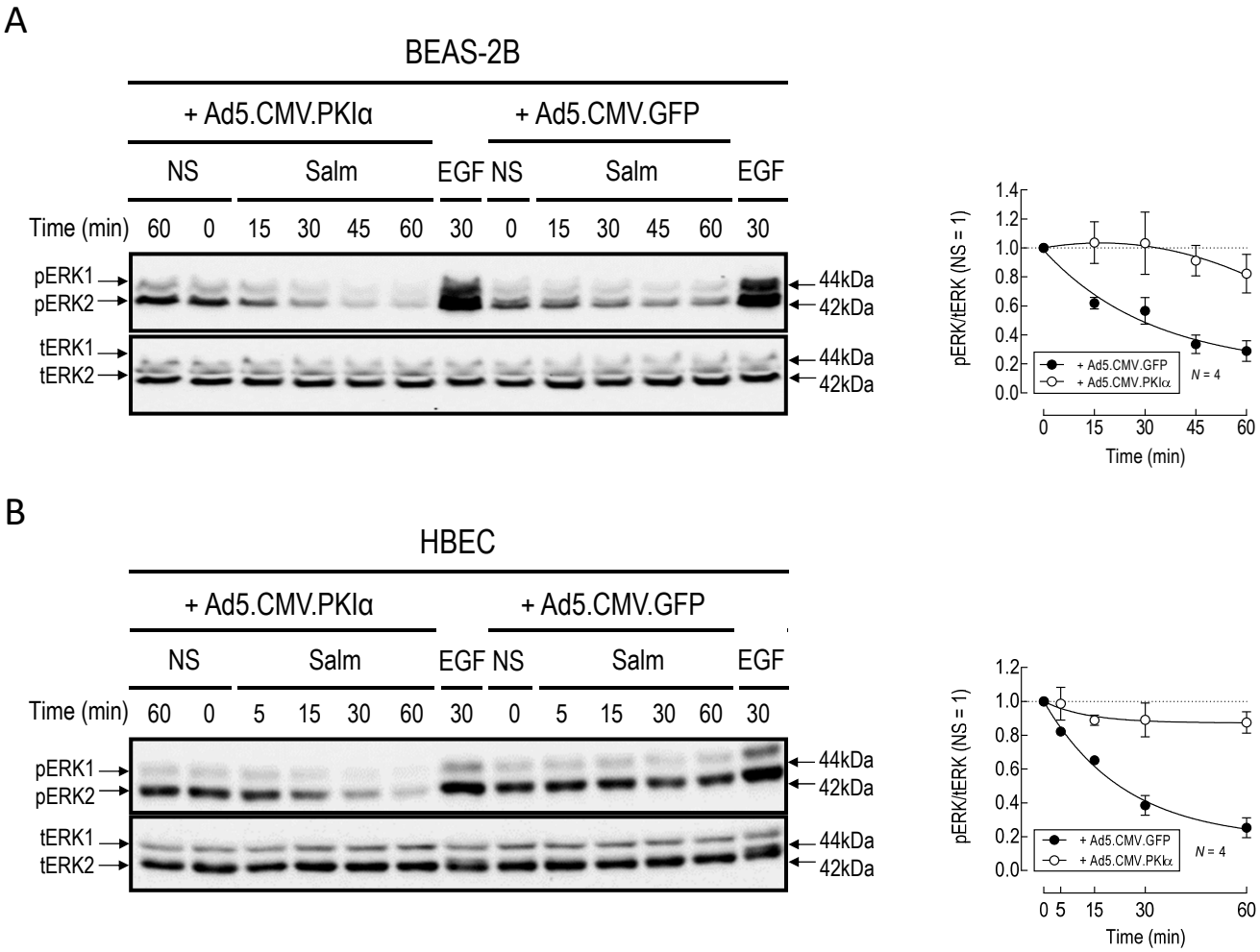
Supplemental Fig. 2. Salmeterol promotes ERK1/2 phosphorylation in HEK293β2 cells by a β_2-adrenoceptor-mediated mechanism. Confluent cells were pre-treated (30 min) with ICI 118,551 (ICI; 1 μM) or vehicle and exposed to salmeterol (Salm; 10 nM) for 1, 5, 15, 30 and 60 min (A). Alternatively, cells were exposed to increasing concentrations of salmeterol for 5 min ranging from -12M to -8M (B). Cell lysates were prepared and subjected to western blotting using antibodies that detect total (t) and phosphorylated (p) ERK1/2. Band volumes were quantified, and pERK1/2 was normalized to tERK1/2 and expressed as fold change from the not-stimulated (NS) baseline level (defined by the dashed horizontal line) at time zero (NS = 1). The concentration of salmeterol that produced half maximal response (pEC_{50}) is also shown. Each panel shows a representative western blot and the mean data (± s.e. mean) of *N* independent determinations.



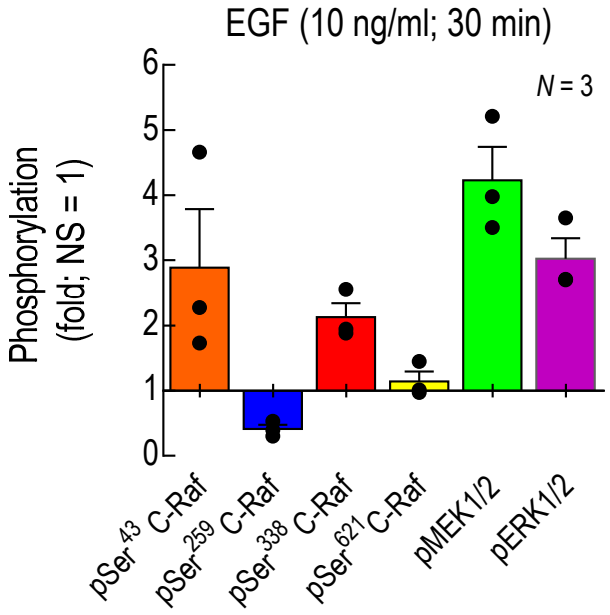
Supplemental Fig. 3. Salmeterol promotes ERK1/2 dephosphorylation in BEAS-2B cells. Confluent cells were either not stimulated (NS) or treated with salmeterol (Salm; 100 nM) for 15, 30, 45 and 60 min (**A**). Alternatively, cells were exposed to increasing concentrations of salmeterol for 60 min ranging from -12M to -6M (**B**). PMA (1 μM; 60 min) and TNFα (10 ng/ml; 60 min) were included as positive controls. Cell lysates were prepared and subjected to western blotting as described in the legend to supplemental figure 2. The time required for pERK1/2 to decline to 50% of the maximal dephosphorylation ($t_{1/2}$) and the concentration of formoterol that produced half maximal response (pEC_{50}) are also shown. Each panel shows a representative western blot and the mean data (± s.e. mean) of *N* independent determinations.



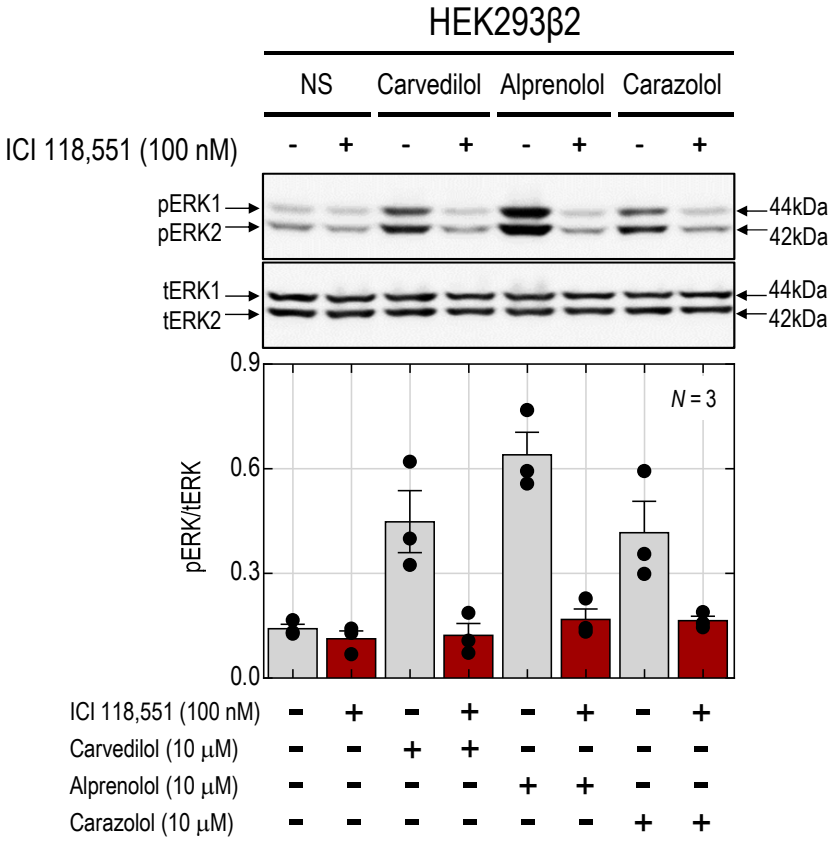
Supplemental Fig. 4. Salmeterol and indacaterol promote ERK1/2 dephosphorylation in HBEC by a β_2-adrenoceptor-mediated mechanism. Confluent cells were treated with salmeterol (Salm; 100 nM; **A** & **B**), indacaterol (Ind; 100 nM; **C**) or vehicle for 15, 30, 45 and 60 min in the absence or presence of ICI 118,551 (ICI, 1 μM)as indicated. The mitogen, EGF (10 ng/ml; 30 min), was included in all experiments as a positive control. Cell lysates were prepared and subjected to western blotting as described in the legend to supplemental figure 2. The time required to reach 50% maximal ERK1/2 dephosphorylation ($t_{1/2}$) is also shown for each agonist. Each panel shows a representative western blot and the mean data (± s.e. mean) of *N* independent determinations. NS, not stimulated.



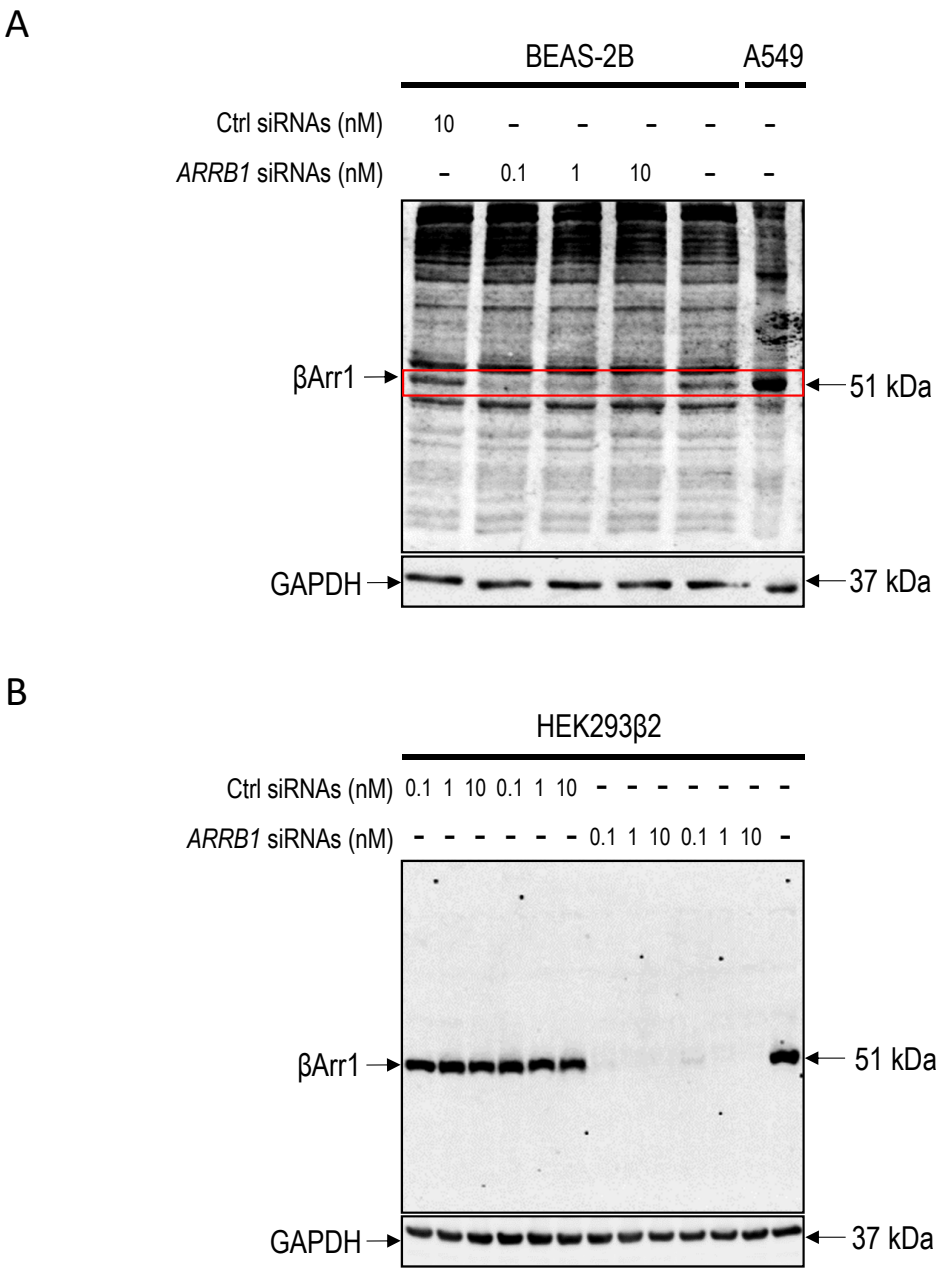
Supplemental Fig. 5. Salmeterol promotes ERK1/2 dephosphorylation in airway epithelial cells in a PKA-dependent manner. BEAS-2B cells (**A**) and HBEC (**B**) were infected (MOI = 25) with Ad5.CMV.PKIα or a control vector (Ad5.CMV.GFP) and treated with salmeterol (Salm; 100 nM) for 15, 30, 45 and 60 min. The mitogen, EGF (10 ng/ml; 30 min), was included in all experiments as a positive control. Cell lysates were prepared subjected to western blotting as described in the legend to supplemental figure 2. Each panel shows a representative western blot and the mean data (± s.e. mean) of *N* independent determinations. NS, not stimulated.



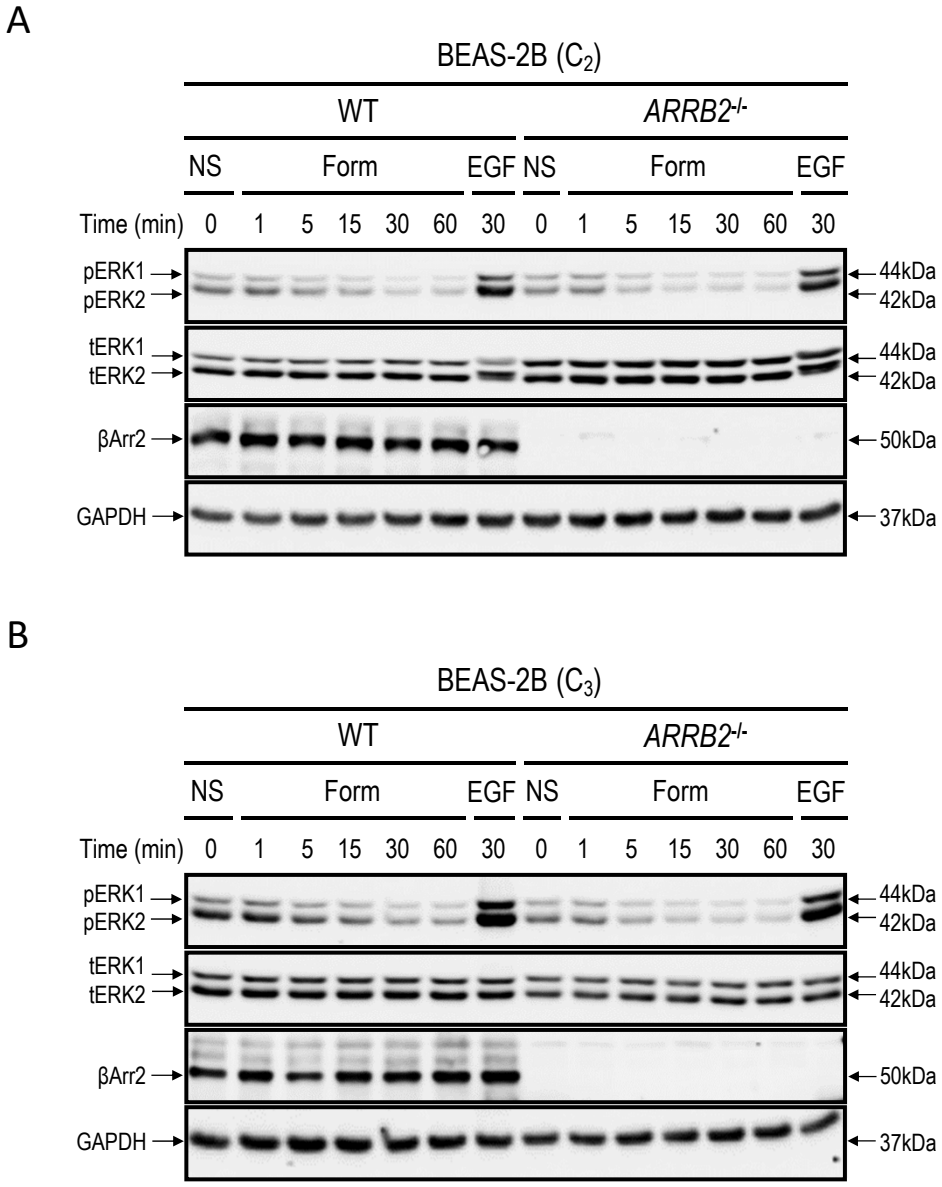
Supplemental Fig. 6. Effects of EGF on the phosphorylation status of C-Raf, MEK1/2 and ERK1/2 in BEAS-2B cells. Cells were treated with EGF (10 ng/ml; 30 min), lysed and subjected to western blotting using antibodies that detect total (t) and phosphorylated (p) C-Raf (at Ser^{43}, Ser^{259}, Ser^{338} and Ser^{621}), MEK1/2 and ERK1/2. Band volumes were quantified and are expressed as a fold change from the not-stimulated (NS) baseline level at 30 min (NS = 1). Data are the mean ± s.e. mean of *N* independent determinations.



Supplemental Fig. 7. Antagonism of carvedilol-, alprenolol- and carazolol-induced ERK1/2 phosphorylation in HEK293β2 cells by ICI 118,551. Confluent cells were either not stimulated (NS) or pre-treated with ICI 118,551 (100 nM; 30 min) and exposed for 5 min to carvedilol, alprenolol or carazolol (each 10 μM). Cell lysates were prepared and subjected to western blotting using antibodies that detect total (t) and phosphorylated (p) ERK1/2. The panel shows a representative western blot and the mean data (± s.e. mean) of *N* independent determinations.

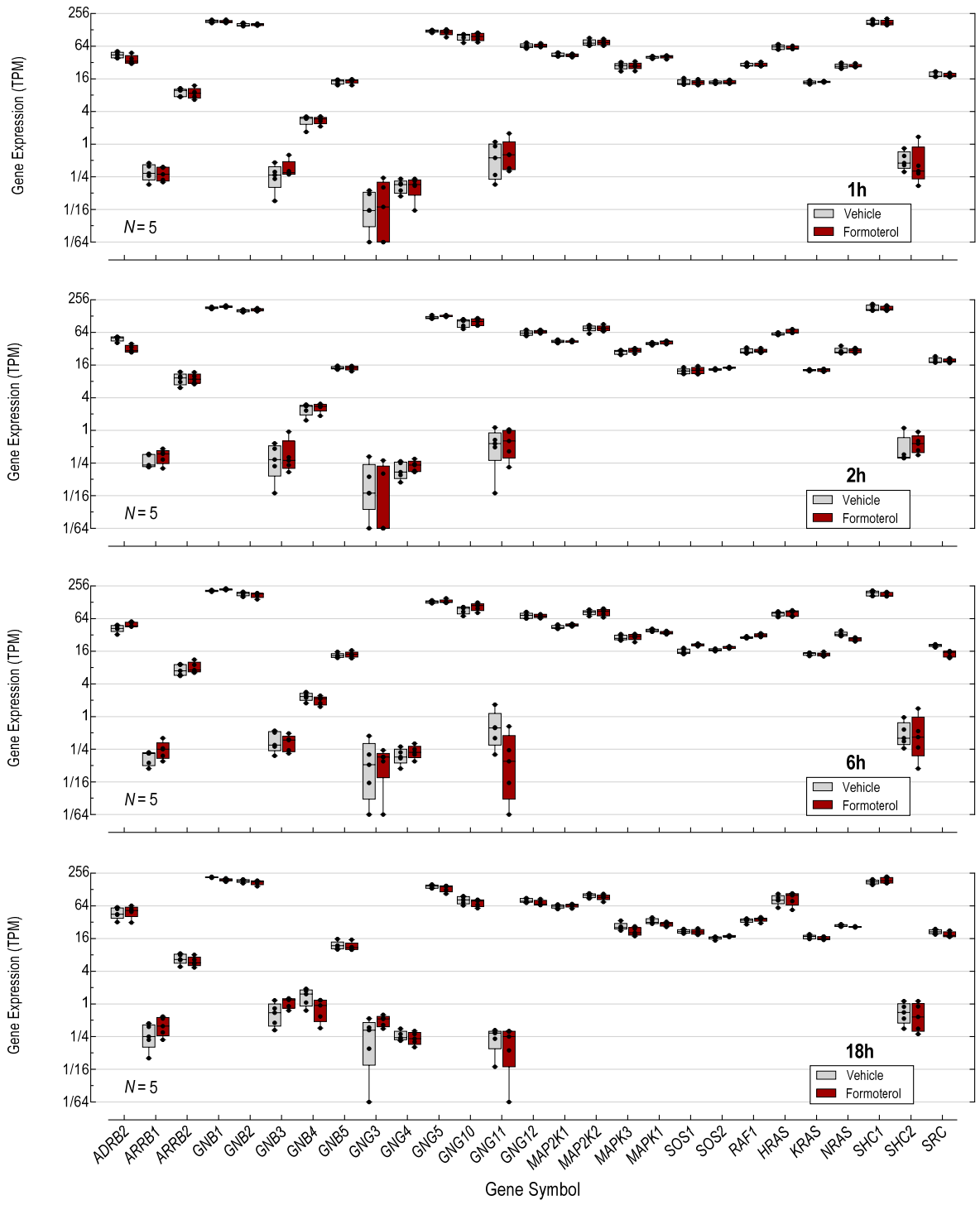


Supplemental Fig. 8. Identification of β-arrestin 1 by western blotting. BEAS-2B cells (**A**) and HEK293β2 cells (**B**) were left untreated or transfected with control and *ARRB1*-targeting siRNAs at 0.1, 1 and 10 nM as indicated. Cell lysates were prepared and subjected to western blotting using an anti-β-arrestin 1 antibody. Strong bands of β-arrestin 1 (βArr1) immunoreactivity were detected in HEK293β2 cells; these migrated at 51 kDa and their intensity was markedly reduced in cells transfected with the *ARRB1*-targeting siRNAs. Using the same antibody, relatively little β-arrestin 1 was present in BEAS-2B (demarked by the red rectangle) and was only visualized using the ultrasensitive chemiluminscent substrate, SignalFire Elite™. Nevertheless, expression was reduced in cells transfected with the *ARRB1*-targeting siRNAs. A549 cells were used as a positive control.



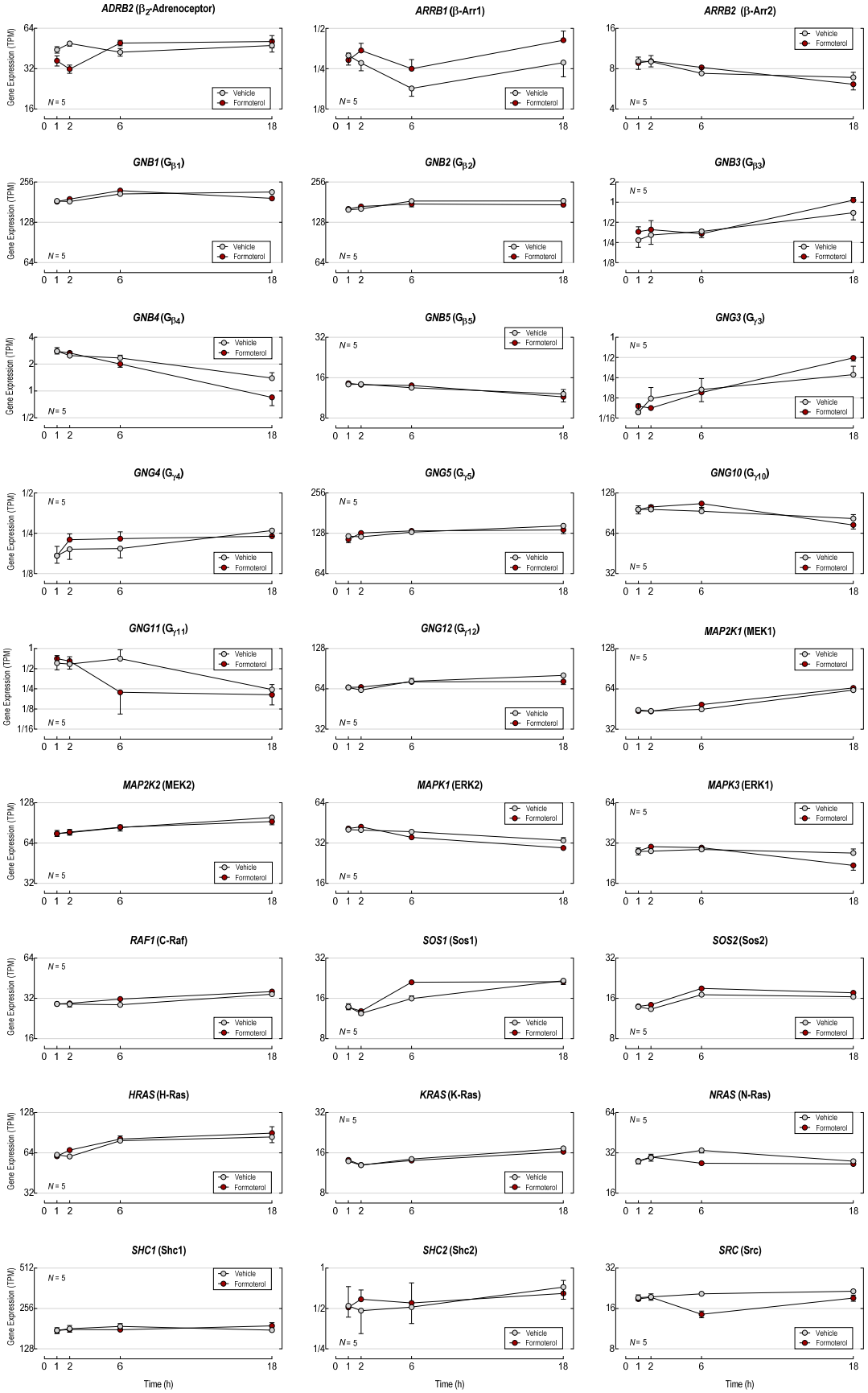
Supplemental Fig. 9. Confirmation of *ARRB2* knockout in clonal BEAS-2B cells and the impact on formoterol-induced ERK1/2 dephosphorylation. Three clones (C_1, C_2 and C_3) deficient in β-arrestin 2 (βArr2) were generated by CRISPR/Cas9 gene editing and identified by western blotting. The deletion was subsequently confirmed by PCR genotyping and Sanger sequencing (Supplemental Fig. 1). Visual inspection of the blots in the top tracks in **A** (C_2) and **B** (C_3) shows that ERK1/2 dephosphorylation produced by formoterol (Form; 1 nM) occurred more rapidly in *ARRB2*$^{-/-}$ cells than in their parental counterparts. The corresponding data on C_1 and quantification of the impact of the deletion on ERK1/2 dephosphorylation are provided in Fig. 8G. The mitogen, EGF (10 ng/ml; 30 min), was included in all experiments as a positive control.

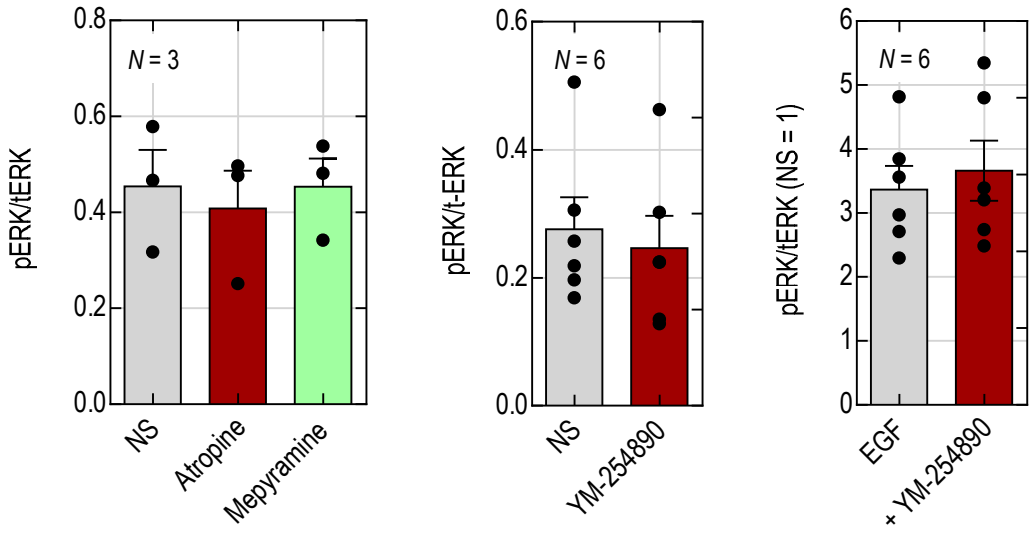
A



Supplemental Fig. 10. Lack of effect of formoterol on the expression of genes in HBEC that are implicated in β_2-adrenoceptor-mediated ERK1/2 phosphorylation in HEK293 and related cell systems. RNA was extracted from HBEC treated with formoterol (1 nM) and vehicle for 1, 2, 6 and 18 h and subjected to RNA-sequencing. Genes expressed at a TPM $\geq$ 0.3 at any time-point were considered biologically meaningful and are displayed on a $\log_2$ scale relative to all genes at 1, 2, 6 and 18 h (**A**) and on a gene-by-gene basis as a function of time (**B**, see next page). Data are presented as box and whisker plots, with each replicate shown as a black circle, and as the mean ± s.e. mean of *N* independent determinations as indicated.

B


ADRB2 (β2-Adrenoceptor)
ARRB1 (β-Arr1)
ARRB2 (β-Arr2)
GNB1 (Gβ1)
GNB2 (Gβ2)
GNB3 (Gβ3)
GNB4 (Gβ4)
GNB5 (Gβ5)
GNG3 (Gγ3)
GNG4 (Gγ4)
GNG5 (Gγ5)
GNG10 (Gγ10)
GNG11 (Gγ11)
GNG12 (Gγ12)
MAP2K1 (MEK1)
MAP2K2 (MEK2)
MAPK1 (ERK2)
MAPK3 (ERK1)
RAF1 (C-Raf)
SOS1 (Sos1)
SOS2 (Sos2)
HRAS (H-Ras)
KRAS (K-Ras)
NRAS (N-Ras)
SHC1 (Shc1)
SHC2 (Shc2)
SRC (Src)
Gene Expression (TPM)
Vehicle
Formoterol
N = 5
Time (h)



Supplemental Fig. 11. Lack of effect of receptor antagonists and Gq inhibition on basal and EGF-induced ERK1/2 phosphorylation. Confluent cells were either not stimulated (NS) or treated (60 min) with atropine, mepyramine or YM-254890 (all 1 μM). Alternatively cells were pre-treated (60 min) with YM-254890 and then exposed to EGF (10 ng/ml) for 30 min. Cell lysates were prepared and subjected to western blotting using antibodies that detect total (t) and phosphorylated (p) ERK1/2. pERK1/2 levels are expressed as either a ratio to tERK1/2 or as a fold change from the not-stimulated baseline level at 90 min (NS = 1) as indicated. Data are the means ± s.e. mean of *N* independent determinations.